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Chapter

Application of PCR Technique to Detect Polymorphism of the KRTAP1.1 Gene in Three Sheep Breeds - A Review

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Abstract

The quality of wool and pelt products depends on the quality of the wool and pelt grown on farm. Genes coding for the proteins involved in the structural components of wool fibre; keratin intermediate filaments (KRTs) and keratin keratin-associated proteins (KAPs) have been extensively researched. The KAPs form a matrix in which the KRTs are embedded. In sheep, KRTAP1.1 (previously B2A) is one of the four genes encoding proteins that make up the KAP1.n family. The ovine KRTAP1.1 gene is clustered with the KRTAP1.3 and KRTAP1.4 genes on chromosome 11. In this chapter, the Polymerase Chain Reaction (PCR) – Applied Fragment Length Polymorphism (AFLP) typing method used to detect polymorphism in the KRTAP1.1 gene is reviewed. Three length variation KRTAP1.1 alleles; named A, B and C, of the lengths 341 base pair, 311 base pair and 281 base pair, respectively have been reported in three sheep breeds; Romney sheep of New Zealand, Merino sheep of New Zealand and Swakara sheep of Namibia. Genetic variation within the KRTs and KAPs can be further exploited to determine as to whether such variation impacts on wool quality. The presence of genetic variation within KRTs and KAPs offers opportunities for the development of gene markers affecting wool and pelt quality traits.

Keywords: genetic variation, KAPs, KRTs, KRTAP1.1, PCR, pelt, sheep, wool

1. Introduction

Domesticated Sheep (*Ovis aries*) are a major source of meat, wool, milk, and pelts/skin or fur, around the s the generic name of hair from animals such as goat, camel, vicuna, alpaca, angora rabbit and yak [1]. Wool grows from follicles on the sheep's skin, similar to the way that hair grows on human skin. Wool fibres are resistant to sunlight, ultraviolet radiation, heat and fire [2]. Because of the unique attributes of wool (excellent insulative properties, breathability and fire resistance [2], the fibre is widely used for clothing, bedding, carpets and other interior textiles.

High quality pelts are produced from Swakara sheep of Namibia, although there are other pelt-producing sheep breeds. Swakara is a fat-tailed sheep that is very hardy and well adapted to arid conditions and mainly kept for the production

of pelts [3–6], though it may also be reared for meat and wool. Originating from Uzbekistan in Central Asia, and imported into Namibia in 1907 (as Karakul sheep then) [3], the intensive research and strategic breeding programmes which were subjected to Karakul sheep in Namibia has resulted in the production of a unique breed named Swakara in Namibia [6]. Swakara pelt production is an exclusive industry that produces outstanding quality pelts characterised by short hair, exceptional patterns and better hair texture [7]. The main product produced from Swakara pelt is high quality leather apparel of various colours that is sought after in the fashion industry. Other accessories, such as hand bags, carpets, shoes, car seat covers, belts are processed.

Wool fibre is made up of three main structures: the cortex, cuticle, and in some coarse wools, the medulla [8]. The cortical cells comprise 90% of the wool fibre, and are responsible for the major physical properties of wool fibre [9]. The cortex consists of the microfibrils, made up of keratin intermediate-filament proteins (KRTs) and embedded in a matrix of keratin intermediate-filament-associated proteins (KAPs) [8, 11, 12] through disulphide cross-linkages [12, 13]. The matrix consists of KAPs, and is divided into three groups based on their amino acid compositions; high-sulphur (HS), ultra-high sulphur (UHS) and high-glycine-tyrosine (HGT) KAPs [8, 14].

The HS KAPs occur at relatively high concentrations in the paracortex when compared to the orthocortex [15], and are encoded by five multigenes families referred to as KRTAP1.n, KRTAP2.n, KRTAP3.n, KRTAP11.n and KRTAP13.n [12]. The HS KAPs are highly conserved at both the amino acid and nucleotide sequence levels [16]. However, there is a consecutively repeated decapeptide unit (QTSCCQPTSI), which varies in its frequency between the HS KAPs [17]. This decapeptide occurs between four times in the KRTAP1.1, three times in the KRTAP1.2, twice in the KRTAP1.3 and five times in the KRTAP1.4 protein [18]. The KAP1.n family is composed of four known proteins, referred to as KAP1.1, KAP1.2, KAP1.3 and KAP1.4, previously referred to as B2A, B2B, B2C and B2D, respectively. The genes that code for the proteins making up the KAPl.n family lack introns and they usually occur in gene clusters [19]. The majority of genes coding for the KAPs have a conserved 18-bp sequence that varies slightly, immediately 5′ to the initiation codon [20, 21]. This suggests that KAPs evolved from a common ancestor, and that their expression may have elements in common.

The KRTAP1.1 gene has been mapped to ovine chromosome 11, clustering with other KRTAP genes [22]. KRTAP1.1 has been reported to be polymorphic in Romney sheep breed of New Zealand [18, 23]; Merino sheep breed of New Zealand [24, 25] and Swakara sheep breed of Namibia [26].

Furthermore, the region spanning the KRTAP1.1/KRTAP1.3/KRT33A loci on ovine chromosome 11 has been associated with variation in wool staple strength in Romney sheep [27].

A number of typing methods have been developed to detect polymorphism in genes that code for traits of economic importance in sheep. These methods are used depending on the specific gene being studied, and the resources available to researchers. In this chapter, the PCR – Applied Fragment Length Polymorphism (AFLP) typing method used to detect polymorphism in the KRTAP1.1 gene is reviewed. Other techniques used to identify polymorphism in keratin genes include PCR - Single strand conformational polymorphism (PCR-SSCP) and PCR - Restriction Fragment Length Polymorphism (PCR-RFLP).

Many studies have described genetic variation within genes that code for the KRTs and the KAPs using PCR-SSCP, including those of Gong *et al*., [28–31], Rogers *et al*., [18], Itenge-Mweza *et al*., [24], Chai *et al.,* [32].

Similarly, many studies have described genetic variation within genes that code for the KRTs and the KAPs using PCR-RFLP. The RFLP patterns were obtained by cutting the KAP1.3 locus defining a 598 bp amplimer using *Bsr* I restriction enzyme in sheep breeds by Xu *et al.,* [33], Chen *et al.,* [9], Kumar *et al*., [34], Mahajan *et al*., [35] and Meena *et al.,* [36]. Parsons *et al*., [37] reported a diallelic polymorphism at the KAP6 locus using *Bam*HI PCR-RFLP to give alleles designated A1 (24.5 kb) and A2 (14.1 kb). Rogers *et al*., [38] reported a di-allelic polymorphism at the KRT33A, formerly known as KRT1.2 defining a 480 bp amplimer in Romney sheep, while Arora *et al.,* [39] found three genotypes (MM, MN, NN) at the KRT33A locus defining 480 bp amplimer in Indian native sheep breeds. Furthermore, Kumar *et al.,* [34] reported three KRT33A genotypes (MM, MN, NN) in Patanwadi and Nali sheep breeds. McLaren *et al*., [22] reported two alleles at the KRT83, formerly known as KRT2.10 locus using a *Bsr* DI.

2. Polymerase chain reaction (PCR)-agarose gel electrophoresis

2.1 Polymerase chain reaction

Developed by Kary Mullis in 1983, Polymerase Chain Reaction (PCR) is a molecular biology technique that is used to produce relatively large numbers of DNA molecules from very small quantity or poor quality. The reaction involves the amplification of a specific segment of the template DNA. A very important requirement is that the sequence of nucleotides on either side of the sequence of interest must be known, so that primers on either side of the sequence of interest can be designed [40]. Primers are short, single-stranded DNA sequence, typically about 18–30 nucleotides in length, that are used as a starting point in DNA synthesis and define the region of the DNA to be amplified. Primers are also referred to as oligonucleotides. In addition to the template DNA and primers, other ingredients needed for the PCR reactions are the DNA polymerase, all four deoxyribonucleotides (dNTP) and magnesium ion (Mg^{2+}). The DNA polymerase used is usually *Taq* Polymerase, isolated from hot springs bacterium, *Thermus aquaticus*, which can withstand the denaturing temperatures [40]. The cycling can be continued without interruption in PCR machines that are simply programmable water baths that accurately and rapidly can change the water temperature that surrounds the reaction mixture [40]. Up to 96 samples can be processed at a time.

2.2 Agarose gel electrophoresis

Gel electrophoresis is a technique by which charged molecules are separated according to their size, by moving through a gel while an electric current is being applied [41]. Agarose is a macromolecular substance that is derived from the cell walls of a number of genera of red algae, such as *Gelidium* and *Gracilaria* [42]. It can be purified to a whitish granular powder which, when mixed with water and heated, it sets like a jelly. This is called a gel and it is used as a molecular sieve for the DNA molecules that can be characterised by both charge and size [41]. DNA has a negative charge due to the negative charge of its phosphate groups attached to the 5′ carbon of one nucleotide and the 3′ carbon of the next nucleotide. When put in solution and an electric field is applied, DNA fragments move from the negative (black) terminal to the positive (red) terminal because of the net negative charge in solution [41]. The movement of charged molecules is called migration. DNA is loaded into pre-cast wells in the gel and a current applied.

The speed that the DNA travels through a gel is inversely proportional to the size of the DNA. A molecular weight marker (MM) also known as DNA marker or size standard is often included on the gel to give an indication of the fragment size. The fragments in the MM are of a known length, and can therefore be used to approximate the size of the fragments in the samples.

Smaller molecules migrate through the gel more quickly than large DNA molecules, as they are less physically restrained by the gel matrix and therefore travel further than larger fragments that migrate more slowly and will therefore travel a shorter distance. As a result, the molecules are separated by size.

Agarose gel electrophoresis can be affected by the following factors:

- The size of DNA molecule
- The percentage of agarose, which affects the sieving of the DNA molecules.
- The voltage applied during the electrophoresis, which cause the DNA molecules to move.
- The type of agarose
- The electrophoresis buffer.

To make a gel, agarose powder is mixed with a running buffer and heated to a high temperature until all of the agarose powder has melted. The most common gel buffers are TBE buffer (89 mM Tris, 89 mM orthoboric acid, 2 mM Na_2EDTA) and TAE (Tris-acetate-EDTA).The main difference between the two buffers is that TAE buffer has better conductivity than TBE. Therefore, DNA fragments migrate faster in TAE buffer than TBE. Nonetheless, TBE buffer supports better agarose cross-linkage, and better resolution of large DNA fragments is obtained better in TBE buffer and better resolution of smaller DNA fragments in TAE buffer.

The molten gel is left to cool down, and then poured into a gel casting tray before it solidifies. To make wells for the sample, a "comb" is placed at one end of the casting tray. The gel is left to set for about 30 minutes, and the comb is removed. The gel is placed in a gel box, also called electrophoresis tank and can be used immediately, or wrapped in plastic wrap and stored at 4 °C until use [43]. Enough running buffer is added into the tank to cover the surface of the gel. It is important to note that the same running buffer is used as the one used to prepare the gel [43].

The DNA samples are mixed with loading dyes (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) prior to loading them into the wells of a gel. The loading dyes have three main functions; firstly, they add density to the sample, allowing it to sink into the gel. Secondly, they provide colour and therefore helps to track how far DNA sample has travelled, and finally, they move at standard rates through the gel, allowing for the estimation of the distance that DNA fragments have migrated. Agarose gel electrophoresis is the most effective way of separating DNA fragments of varying sizes ranging from 100 to 25,000 bp [43]. **Table 1** shows the typical agarose gel concentration used to resolve DNA fragments. It is worth noting that base pairs less than 500 are better separated using polyacrylamide gel, with gel percentage between eight to 20%.

After the electrophoresis is complete, the molecules in the gel can be stained with an appropriate dye to make them visible. This may be performed either with Ethidium bromide (EtBr) to a concentration of 0.5–1 μg/ml, silver staining, or coomassie blue dye. Though other methods may also be used to visualise the separation, EtBr is the most common reagent used to stain DNA in agarose gels. It works

DNA fragment (bp)	Agarose gel percentage (%)
1000–30,000	0.5
800–12,000	0.7
500–10,000	1.0
400–7,000	1.2
200–3,000	1.5
100–2,000	2.0

Table 1.
Typical agarose gel concentration for resolving DNA fragments.

by intercalating itself in the DNA molecule in a concentration dependent manner. This allows for an estimation of the amount of DNA in any particular DNA band based on its intensity [43].

To visualise the DNA, the gel is stained with a fluorescent dye that binds to the DNA, and is placed on an ultraviolet transilluminator which will show up the stained DNA as bright bands. EtBr is the most common reagent used to stain DNA in agarose gels. When exposed to ultraviolet light, electrons in the aromatic ring of the EtBr molecule are activated. EtBr works by intercalating itself in the DNA molecule in a concentration dependent manner. This allows for an estimation of the amount of DNA in any particular DNA band based on its intensity.

2.3 Length variation of the KRTAP1.1 gene

2.3.1 KRTAP1.1 primers

KRTAP1.1 primers were designed by Itenge-Mweza *et al*., [24], designed to amplify a 311 bp fragment of the KRTAP1.1 gene, based on a published gene sequence [10]; GenBank accession number X01610). The primers were: KRTAP1.1up 5'-CAA CCC TCC TCT CAA CCC AAC TCC-3′ and KRTAP1.1dn 5′- CGC TGC TAC CCA CCT GGC CAT A-3′.

2.3.2 Amplification of KRTAP1.1 gene using PCR

PCR amplifications was performed by process discussed by Itenge-Mweza *et al*. [24] using Merino Sheep; Itenge [25], Rogers *et al*., [18] using Romney sheep and Nyoni *et al.*, [26] using Swakara sheep. Amplification consisted of 1 min denaturation at 95 °C, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 65 °C for 1 min and extension at 72 °C for 1 min, with a final extension of 72 °C for 7 min. Amplimers were stored at 4 °C until they were subjected to agarose gel electrophoresis, described in Itenge-Mweza *et al*., [24] and Rogers *et al*., [18].

2.3.3 Amplified fragment length polymorphism (AFLP) in KRTAP1.1

KRTAP1.1 amplicons visualised by electrophoresis in 2% SeaKem® LE agarose (FMC Bioproducts) gels, and a photograph was taken for records [24]. A study by Rogers *et al*., [18] was the first to report length polymorphism in the KRTAP1.1 gene of Romney sheep. Three variants were identified, designated Aα, Aβ and Aγ, (GenBank accession numbers L33885, L33886 and L33887, respectively), and ranging from largest to smallest. The frequency of the Aα, Aβ and Aγ alleles in 19 unrelated Romney sheep was calculated to be 0.13, 0.71 and 0.16, respectively.

A study by Itenge-Mweza *et al,* [24] reported three variants of the KRTAP1.1 gene that varied in length in Merino sheep breed of New Zealand. These were designated as A, B and C (**Figure 1**) and differed in part through variation in the length of a conserved 30 nucleotide repeat sequence; GenBank accession numbers AY835603, AY835603 and AY835605, respectively). The lengths of the coding sequences of alleles A, B and C reported by Itenge-Mweza *et al*., [24] were 341 bp, 311 bp and 281 bp, respectively, and these compared with the alleles reported by Rogers *et al.,* [18]. Sequence analysis revealed that the length of each of these AFLP bands were 341 bp, 311 bp and 281 bp, respectively (**Figure 2**). Sequence comparison shows that Alleles A and C in Itenge-Mweza *et al*. [24, 25] correspond to those of [17]. On the contrary, Merino allele B (GenBank accession no. AY835604) shows five single nucleotide differences from the sequence by Rogers *et al*., [18], suggesting a possible fourth KRTAP1.1 allele. Sequencing analysis also confirmed that the length variation detected in Itenge-Mweza *et al*., [24] was the result of an insertion or deletion of a 30 bp region of the sequence (**Figure 2**).

Three length polymorphism alleles within the KRTAP1.1 gene were identified by Nyoni *et al*., [26] in Swakara sheep, designated as A, B and C. The band sizes of the alleles (A-C) estimated from the molecular marker included on a 2% agarose gel indicated that they were similar to the sizes reported by Itenge-Mweza *et al*., [24], of 341 bp, 311 bp and 281 bp, respectively. Sequencing results of the three alleles revealed that the A allele had 100% homology with GenBank accession number AY835603. Allele B had 100% homology with GenBank accession numbers FJ169479 and FJ169479.1, while allele C had 100% homology with GenBank accession number MG641066.

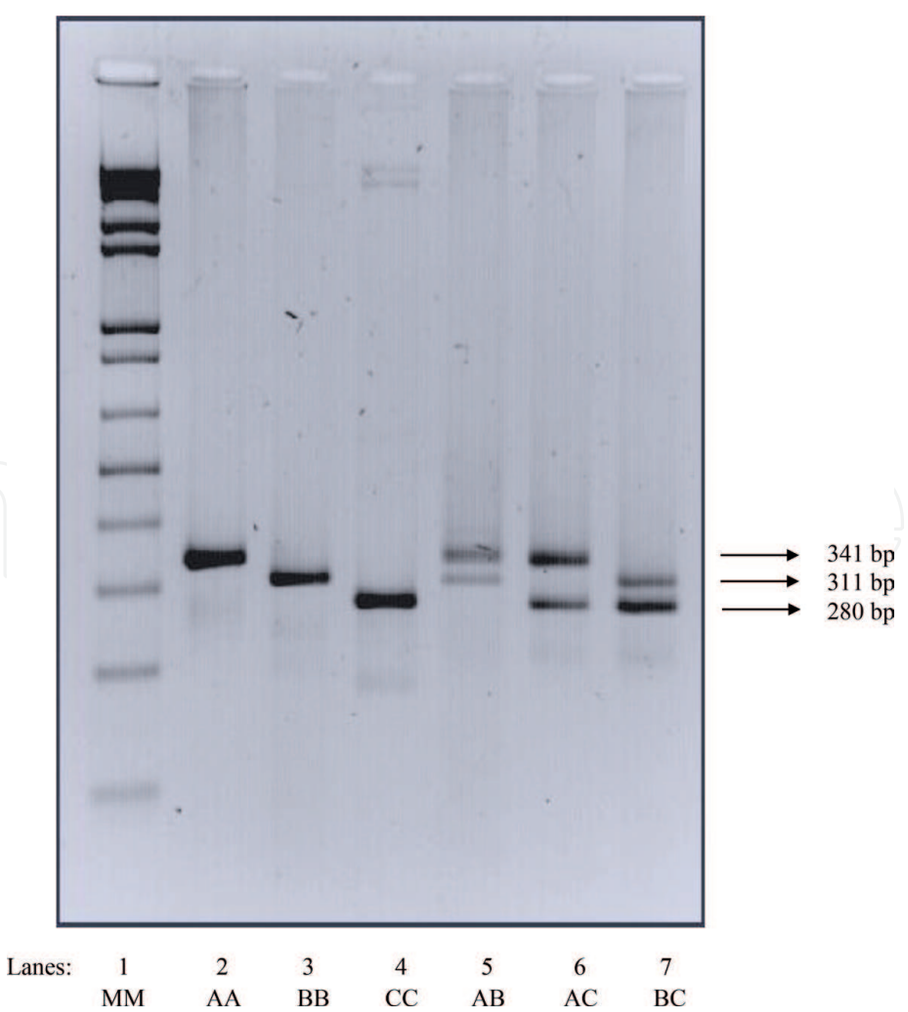

Figure 1.
Length polymorphism within the KRTAP1.1 amplimer, separated in a 2% agarose gel electrophoresis at a constant 10 Vcm^{-1} for 90 min. Lane 1 contains a molecular weight marker (MM) [32].

```
A_(AY835603) CAACCCTCCTCTCAACCCAACTCCTGACACCATGGCCTGCTGTTCCACCA     50
B_(AY835604) --------------------------------------------------     50
C_(AY835605) --------------------------------------------------     50
α_(L33885)   --------------------------------------------------     50
β_(L33886)   --------------------------------------------------     50
γ_(L33887)   --------------------------------------------------     50
X01610       --------------------------------------------------     50

A_(AY835603) GCTTCTGTGGATTTCCCATCTGTTCCACTGGTGGGACCTGTGGCTCCAGT    100
B_(AY835604) --------------------------------------------------    100
C_(AY835605) --------------------------------------------------    100
α_(L33885)   --------------------------------------------------    100
β_(L33886)   ----------------------c---------------------------    100
γ_(L33887)   --------------------------------------------------    100
X01610       --------------------------------------------------    100

A_(AY835603) CCCTGCCAGCAGACCTGCTGCCAGACCAGCTGCTGCCAGCCAACCTCCAT    150
B_(AY835604) ----------c-----------------------------------.....    145
C_(AY835605) ----------c-----------------------------------.....    145
α_(L33885)   --------------------------------------------------    150
β_(L33886)   ----------c-----------------------------------.....    145
γ_(L33887)   ----------c-----------------------------------.....    145
X01610       ----------c-----------------------------------.....    145

A_(AY835603) CCAGACCAGCTGCTGCCAGCCAACTTCCATCCAGACCAGCTGCTGCCAAC    200
B_(AY835604) ........................----------------------------    170
C_(AY835605) ........................----------------------------    171
α_(L33885)   --------------------------------------------------    200
β_(L33886)   ........................----------------------------    170
γ_(L33887)   ........................----------------------------    171
X01610       ........................----------------------------    170

A_(AY835603) CGATCTCCATCCAGACCAGCTGCTGCCAGCCAACCTCCATCCAGACCAGC    250
B_(AY835604) --------------------------------------------------    220
C_(AY835605) ----.............................----------------    190
α_(L33885)   --------------------------------------------------    250
β_(L33886)   ------t-----------------------------t-------------    220
γ_(L33887)   ----.............................----------------    190
X01610       --------------------------------------------------    220

A_(AY835603) TGCTGCCAGCCAACCTGCCTCCAGACCAGTGGCTGTGAGACGGGCTGTGG    300
B_(AY835604) --------------------------------------------------    270
C_(AY835605) ----------------------------------------c---------    240
α_(L33885)   --------------------------------------------------    300
β_(L33886)   --------------------t-----------------------------    270
γ_(L33887)   ----------------------------------------c---------    240
X01610       --------------------------------------------------    270

A_(AY835603) CATTGGTGGCAGCATTGGCTATGGCCAGGTGGGTAGCAGCG             341
B_(AY835604) ---------------------------------------             311
C_(AY835605) ---------------------------------------             281
α_(L33885)   ---------------------------------------             341
β_(L33886)   ------------------t--------------------             311
γ_(L33887)   ---------------------------------------             281
X01610       ---------------------------------------             311
```

Figure 2.
Comparison of KRTAP1.1 alleles a, B and C (GenBank accession numbers AY835603 - AY835605, respectively) with [11] alleles α, β and γ, (GenBank accession numbers L33885, L33886 and L33887, respectively) and part of the published KRTAP1.1 gene sequence X01610 [32]. Upstream and downstream primers are underlined and the start codon is bolded. Dashes represent similar nucleotide to a allele above and dots represent nucleotide missing in the alleles. Represent chi-like sequences. Represent tandem repeat sequence. It is of note that there is minor sequence variation at the DNA level both within and between the genes.

A major difference between the KRTAP1.1 alleles is the insertion/deletion of a 30 bp nucleotide, which produces a decapeptide (QPTSIQTSCC) in the gene [24]. Interestingly, it was reported by [22, 23, 41] that the inserted/deleted decapeptide is the same as the consecutively repeated decapeptide unit of the other

HS KAPs (KRTAP1.2, KRTAP1.3 and KRTAP1.4. The number of alleles reported in the KRTAP1.2, KRTAP1.3 and KRTAP1.4 genes are two, ten and nine alleles, respectively.

It is not yet clear as to why KRTAP1.1 would have an inserted/deleted decapeptide unit. However, the decapeptide contains two cysteine amino acids. The supply of cysteine is limiting to wool growth [44]. Itenge-Mweza *et al*., [24] stated that analysis of sheep from diverse breeds may reveal even more KRTAP1.1 alleles, and recommended that a new typing method be developed as the amplified fragment length polymorphism (AFLP) does not differentiate between all of the alleles at the KRTAP1.1 locus in sheep. It was also proposed by Gong *et al*., [12] that further investigation using other typing methods, rather than AFLP in the KRTAP1.1 is needed, in order that full extent of variation in this gene is revealed, should it exist.

2.4 Length variation of other wool genes

Other than KRTAP1.1, there are other wool genes that have been reported to contain length polymorphism due to a change in the number of repeat sequences. These include trichohyalin (an important wool follicle protein) [45], and the Type II keratins genes [46, 47].

3. Conclusion

Genetic variation within the genes which make up the structural component of wool fibre can be further exploited to determine as to whether such variation impacts on wool quality. The presence of genetic variation within genes that code for the proteins involved in the structural components of wool fibre offers opportunities for the development of gene markers affecting wool quality traits.

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